

In vivo Anti-Inflammatory and Anti-Arthritic Potential of Ethanolic *Acacia modesta* Extract on CFA-Induced Adjuvant Arthritic Rats

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ABSTRACT

The objective of the present study was to investigate the role of *Acacia modesta* extract whole plant extract (E-AM) in process of inflammation and also in arthritis by using animal model of rat. Thirty-six healthy wister rats were chosen for *in vivo* experiments and divided into six groups. Arthritis was induced in rats using Complete Freund's Adjuvant (CFA) and treated with different concentrations of E-AM (250, 500 or 750 mg/kg), and with standard treatment, piroxicam (10 mg/kg). Antioxidant assays (superoxide dismutase and catalase) and histopathological examination were performed to determine the values of various parameters of inflammation and arthritis. Real-Time PCR technique was applied to analyze the expression levels of different cytokines and interleukins involved in inflammation. Real-Time PCR results showed a down-regulation of TNF- α , COX-2, IL-6, IL-1 β , NF- κ B and up-regulation of IL-4, IL-10 and I- κ B genes. Levels of antioxidants, superoxide dismutase and catalase were determined to be significantly higher ($p < 0.05$) in the group treated with E-AM compared to the arthritic group. In addition, histopathological examination indicated reduction in the index of C-reactive protein, rheumatoid factor and bone and cartilage erosion after treatment with E-AM. Collectively, our findings suggest that E-AM extract might have potential to perform anti-inflammatory and anti-arthritic activity which could have beneficial impact for the patients associated with arthritis.

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Authors' Contribution

All author presented the content and designed the study. FA, MS, TM and FH prepared material, collected and analysed data. FA, TM and FH wrote the manuscript. SA edited and approved the manuscript. YH did statistical analysis. TAS critically evaluated the manuscript.

Key words

Arthritis, Inflammation, Histopathology, *Acacia modesta*, CFA

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease and a type of chronic inflammation accompanied by synovial proliferation, massive irreversible destruction of cartilage, bone and systemic side effects including cardiovascular, pulmonary, psychological, and skeletal disorders. Furthermore, clinical manifestations include stiffness, lack of physical mobility, swelling, redness, pain

and joint deformity (Hasan *et al.*, 2015). According to an epidemiological survey, the worldwide prevalence rate of arthritis is 1% of human population and its incidence is more common in females compared to males with a ratio 2.5: 1 (Ahmed and Bader, 2015). Thus, patients with arthritis are immensely affected by the social and psychological issues along with limited physical mobility, thereby compromising the patient's quality of life.

Different therapies are executed including the use of anti-histamines, corticosteroids mainly cortisone injection and diuretics. While, current treatment protocol involves non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs) which slow down the progression of the inflammation associated with RA. Although, drugs are effective in reduction of pain yet unable to provide a complete cure. Additionally, stomach ulcer caused by continuously intake of NSAIDs and steroids is another drawback in treating arthritis with synthetic drugs (Cameron *et al.*, 2009). Arthritis is very

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much excruciating because joints connect bones helping in movement of body parts. Inflammation may affect patient's mobility triggered by swelling, pain and heat. Whereas, uncontrolled inflammation can also enhance C-reactive protein production in the body, a potential risk factor of heart disease. All such factors limit the conventional treatment thus, causing the patients to pursue alternative or herbal treatment. Therefore, it is reported that, about 60 to 90% rheumatoid arthritis patients prefer seeking complementary and alternative medication (Soeken *et al.*, 2003). Increased levels of tumour necrosis factor (TNF- α), interleukin 1 and interleukin 6 are mainly involved in affecting the physiological condition and other important cytokines ultimately causing rheumatoid arthritis. Reduction in levels of these cytokines (IL-1 and IL-6, TNF- α) by E-AM would provide a potential mechanism of E-AM in patients suffering from rheumatoid arthritis (Alvaro-Gracia *et al.*, 1991).

Phytonutrients are basically the plants derived nutrients which may exert potential biological activity to fight various health related issues (Kumar and Karambir, 2012). In a recent research, *Punica granatum* possessing flavonoids and phenolic compounds showed anti-arthritis activity (Gautam *et al.*, 2018). Gum Arabic enhanced the anti-inflammatory cytokine IL10 while diminished the levels of C-reactive proteins in arthritic group (Kamal *et al.*, 2018). In another research, *Asphodelus tenuifolius* is proven traditionally effective in treatment of pain in rheumatoid arthritis and in causing reduction in its symptoms (Saleem *et al.*, 2021). *Acacia modesta* is a deciduous small to medium sized tree belongs to the family, Fabaceae with the common name "phulai" and local name palosa. Geographically, its distribution is concentrated in Pakistan, Afghanistan, and India. It is reported that different parts of *Acacia modesta* exhibited diverse pharmacological activities like anti-inflammatory, anti-bacterial, anti-fungal, anti-hyperglycemic, analgesic, anti-termite, antioxidant, spasmolytic and phototoxic activity. Whereas, its phytochemical analysis revealed that many bioactive compounds including flavonoids, alkaloids, terpenoids, tannins as well as non-protein amino acids (cyclitols) and fixed oils can be isolated from it. Among flavonoids, major groups are flavones, isoflavones, flavanones, flavonols, dihydroflavonols, catechin, chalcones and anthocyanidins, while it also constitutes quercetin and kaempferol (Nobili *et al.*, 2009). We previously studied anticancer activity of *Acacia modesta* and found it a good anticancer plant extract (Abid *et al.*, 2020). However, the present study aimed at pharmacological as well as toxicological evaluation of *Acacia modesta* in order to access their medicinal importance in the pathologies of arthritis.

MATERIALS AND METHODS

Laboratory animals and ethical approval

Healthy wistar rats weighing 200-250g were purchased from University of Lahore, Lahore. These animals were kept in steel cages, provided with standard laboratory conditions (25°C temperature and 60% humidity). Animals were provided with free rodent diet and water. Adequate provision of all facilities was ensured. The health and well-being of animals was evaluated on daily basis by the attending veterinarian. These animals were acclimatized for 2 weeks to the laboratory conditions before the conduction of experiments. A combination of ketamine and xylazine was used as an aesthetic treatment in the current study. Doses used were; 100mg/kg body weight for ketamine and 5mg/kg body weight for xylazine.

Animal ethics

Experimental protocols were approved by Institutional Ethical Committee, University of Lahore, Pakistan (Approval no; IMBB/UOL/20/416). All the methods were performed in accordance with the relevant guidelines and regulations.

Plant ethics

The use of plants in the present study complies with international, national and our institutional guidelines.

Preparation of plant extract

Extract of *Acacia modesta* (AM), authorized by a taxonomist with voucher no. 3655 was prepared according to our recently published article (Abid *et al.*, 2020; Maqbool *et al.*, 2019). Extract (E-AM) was dried to solid or semisolid mass by placing in a hot air oven at 25°C. Extract was divided into 250,500 and 750 mg doses, respectively.

Quantitative analysis of constituents in E-AM

Ethanollic plant extract was analysed for phenolic and flavonoids contents for quantitative determination using high pressure liquid chromatography (HPLC) according to Kumar and Jain (2015).

Grouping of animals in acute and chronic models

Thirty six rats were divided into 6 groups each containing 6 animals: Untreated group, 2nd (injury group), 3rd (reference group, piroxicam 10mg/kg), 4th, 5th and 6th (treatment groups) with different concentrations of plant extract *Acacia modesta* 250mg/kg, 500mg/kg and 750mg/kg, respectively.

Acute anti-inflammatory models, carrageenan induced oedema

Pre-treatment of plant extract (250, 500 and 750mg/

kg) and piroxicam (10mg/kg) was orally administered to rats. Inflammation was induced after half an h at day “0” using 0.1ml of 1% (1g in 100 ml of distilled water) freshly prepared solution of carrageenan (Sigma Aldrich, Germany) injected in sub-planter area of right hind paw to all groups of rats except normal control. Digital plethysmometer was used to measure paw size at 1,2,3,4 and 5th h interval. Percentage inhibition was determined and calculated as Mean \pm SD (Hassimotto *et al.*, 2013).

Chronic arthritis model, complete Freund's adjuvant (CFA) arthritis

CFA model of chronic arthritis is an important tool in analysis of pharmacological and pathophysiological regulation of inflammatory procedures along with assessing the anti-arthritic role of drugs (Li *et al.*, 2018). Arthritis induction was done by CFA (0.15ml was injected into left paws of sub planter region of all animals except vehicle control group at day 0 and then paw oedema was measured by using digital plethysmometer started at a day 8th until 12th, 16th, 20th, 24th and 28th day. Animals were treated with E-AM and piroxicam from 8th till 28th day (Cui *et al.*, 2019). Arthritic progression was observed on day 8, 12, 16, 20 and 28. Arthritic scoring was done on the basis of macroscopic observation of paw redness, swelling and inflammation (Lin *et al.*, 2014).

Histopathological investigation

Histopathological investigation was done after sacrificing the rats on 28th day and ankle joints were separated. Slides were prepared and fixed with the haematoxylin and eosin (H and E) (Dhouskar *et al.*, 2019). Different parameters such as pannus formation, bone and cartilage erosion were measured according to histopathological scoring method (Zhang *et al.*, 2015).

Haematological and biochemical parameters

Haematological parameters were performed according to Alghadir *et al.* (2020) while the biochemical assays were performed according to Hadi *et al.* (2020).

Real-time PCR

Gene expression levels of various inflammatory cytokines such as TNF- α , IL-6, COX2, IL-1 β , NF κ B1 and antiinflammatory biomarkers such as IL-4 and IL-10 were analysed using real-time PCR according to our previously published method (Akhtar *et al.*, 2020).

Statistical analysis

Data were interpreted by using Graph pad prism 8.0.1. Statistically data were analysed by two-way analysis of variance (ANOVA) and one way ANOVA for determination of differences among each group along with

Tukey's multiple comparison test and represented by mean \pm SEM. $P < 0.05$ was considered statistically significant.

RESULTS

Carrageenan induced paw oedema

Treatment with *Acacia modesta* inhibited paw oedema induced by Carrageenan. Considerable oedema inhibition with E-AM in contrast with untreated control group at time 1st, 2nd, 3rd, 4th and 5th h is shown in Figure 1.

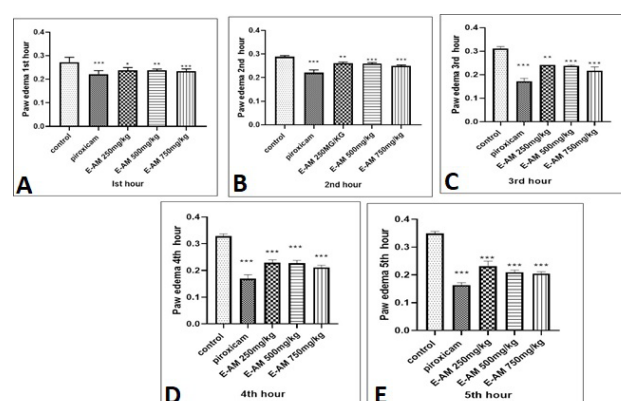


Fig. 1. Effect of different concentrations of ethanolic extract of *Acacia modesta* for different time intervals on paw edema induced by Carrageenan. A showing treatment at 1st h, B is at 2nd h, C at 3rd h, D at 4th h and E at 5th h. ***, **, * showing $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$.

Effect of CFA-induced arthritis on body weight and structure of rat paw

Administration of CFA in sub-planter region of rat paw increased the inflammation and resulted in peak swelling noticed on 8th day. Elevation in paw volume in arthritic control group was observed on day 8 to 28th comparable with normal control and treated group. Percentage inhibition with E-AM was also increased comparable with the standard drug i.e., piroxicam on 28th day, arthritis affects the body weight of rats continuously as inflammation developed. Result indicated that weight loss was more prominent in arthritic control rats as compared to control group from the 8th day to the end of study. On the other hand, plant extract-treated group and piroxicam-treated group appreciably restored the body weight in arthritic rats compared to the arthritic control group from 12th to 28th day as shown in Figure 2. The ethanolic extracts of plant restored body weight of arthritic rats in dose dependent manner. Gross microscopic evaluation of rat paw depicted severe oedema in arthritic rats, less pannus formation and bone erosion in rats treated with E-AM and no oedema in normal control. On 28th day of conducting CFA model, results showed minimal bone erosion with E-AM, less

pannus formation in rats treated with E-AM at 750mg/kg and also reduced vascular degeneration in treatment with E-AM at 500mg/kg and at 750mg/kg which was comparable in dose dependent manner to arthritic control group. All histopathological parameters i.e. bone erosion, pannus formation and vascular degenerative changes were also reduced in arthritic rats as compared to piroxicam shown in [Figure 2](#).

Effect of CFA induced arthritis on liver function, haematological parameters and arthritic index

In case of liver functions; it was noted that induction of arthritis in rats resulted in an increase in ALP, AST and ALT, while after treatment with the plant extract, a significant decrease in the ALP, AST and ALT values was observed as compared to the disease control group alone. Haematological parameters were also determined.

Induction of RA and treatment with the plant extracts and standard Piroxicam had no significant effect on urea and creatinine levels. There was a decrease in haemoglobin (Hb) and an increase in white blood cells (WBCs) and platelets were observed in arthritic rats in comparison with the normal control. It was found that induction of RA in rats resulted in an increase in the Rheumatoid factor (RF). However, treatment with the plant extracts significantly decrease the RF value in rats. In case of arthritic index, the normal control group did not show any swelling throughout the study. The results have shown continuous rise in arthritic index as compared to the normal group. Treatment with the plant extracts and Piroxicam (10mg/kg) effectively reduce the arthritic index in comparison to arthritis control group from day 16 to 28 day end of study as shown in [Figure 3](#).

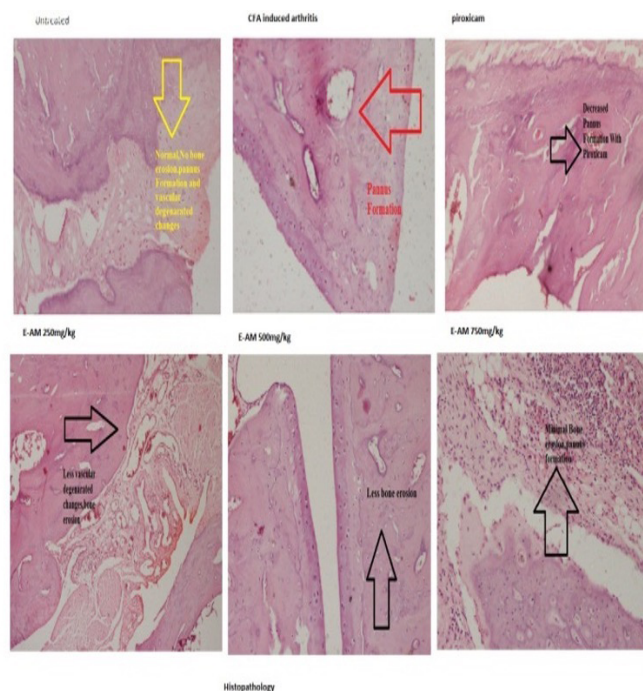
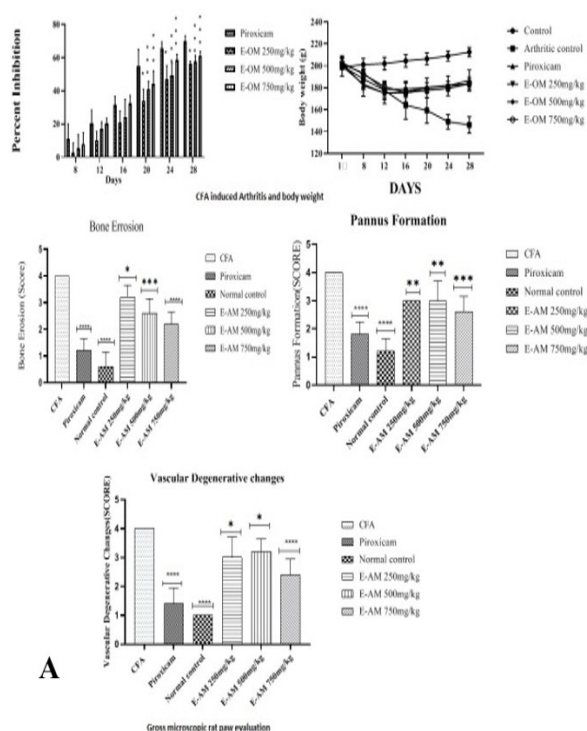


Fig. 2. Effect of different concentrations of ethanolic extract of *Acacia modesta* on carrageenan induced arthritis at different days, body weight, gross microscopic evaluation of rat paw where in case of **1**; CFA induced arthritis and body weight, **A** showing carrageenan induced arthritis at different days and **B** showing treatment group where E-OM with 750mg/kg dose showed an increase in body weight at 28 day as compared with arthritic group. In case of **2**; gross microscopic evaluation of rat paw. **A** showing bone erosion **B** is showing pannus formation **C** is showing vascular degenerative changes in case of **3**; histological appearance of pannus formation, bone erosion and vascular degenerated changes (**A-F**). All histopathological parameters i.e. bone erosion, pannus formation and vascular degenerative changes were also reduced in arthritic rats as compared to Piroxicam. Gross microscopic rat paw evaluation at end of CFA model depicts severe edema in arthritic rats, less pannus formation and bone erosion in rats treated with E-AM. ***, **, * showing $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$.

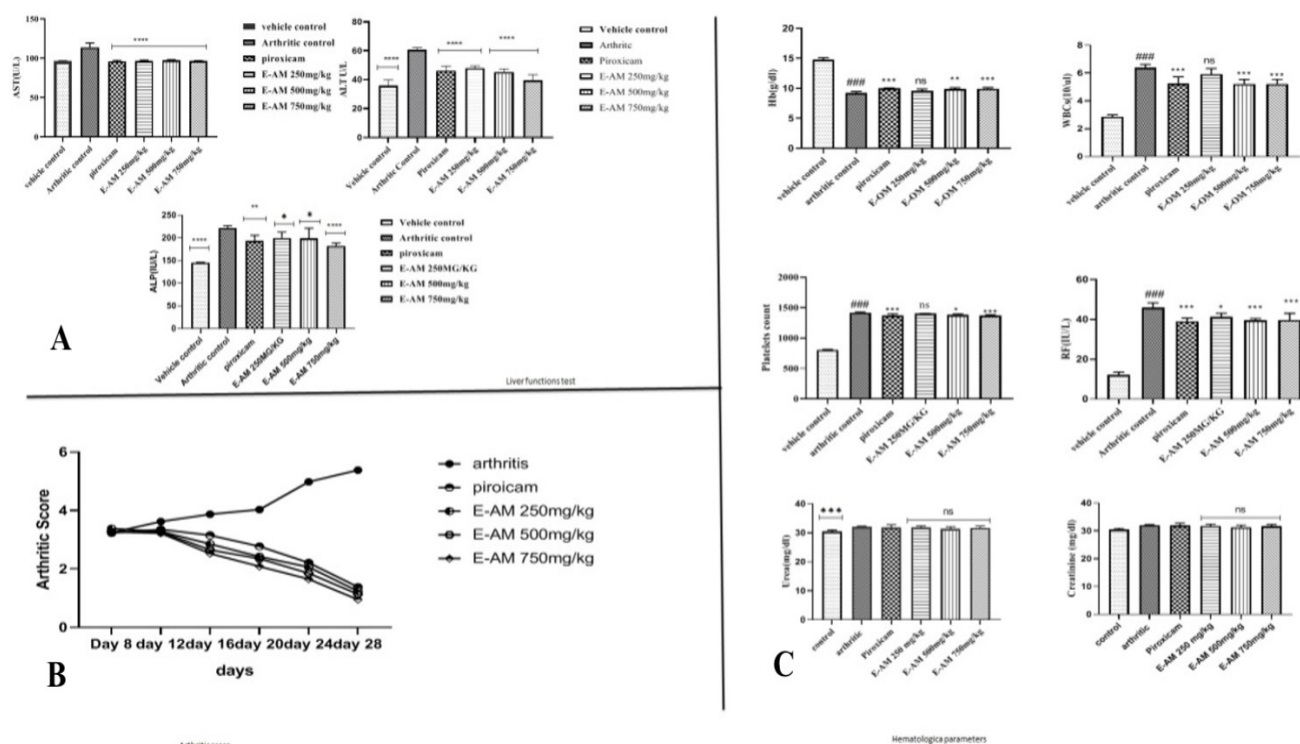


Fig. 3. Effect of different concentrations of ethanolic extract of *Acacia modesta* on liver function tests (A), hematological parameters (B) and arthritic score (C). A, Liver markers ALP, ALT and AST were calculated by one-way ANOVA and Dennett's multiple comparison test. E-AM treated extracts showed a normal ALP, ALT and AST levels. B, Hematological parameters E-AM showed ameliorating effect on hematological parameters at a dose of 750 mg/kg. C, arthritic index the normal control group did not show any swelling throughout the study. Treatment with the E-AM and piroxicam effectively reduce the arthritic index in comparison to arthritis control group from day 16 to 28. The maximum arthritic index observed at 28th day that was restored by E-AM at 750mg/kg compared with low doses. ***, **, * showing $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$.

Effect of E-AM on gene expression and oxidative stress level

Gene expression analysis of several inflammatory biomarkers was evaluated following 28 days of treatment in wistar rats for mRNA expression. IL-10 and IL-4 expression were significantly reduced in arthritic control group. However, it was significantly up-regulated in groups treated with all the concentrations of E-AM (750mg/kg, 500mg/kg, 250mg/kg) and piroxicam treated group when compared to the arthritic control group. While COX-2, NF- κ B1, TNF- α and IL-6 were significantly up-regulated in arthritic control group. However, these were significantly attenuated by treatment with E-AM and piroxicam. In case of anti-oxidative potential, the findings of CFA induced oxidative stress and the ameliorating effect of ethanolic extract of plants was evaluated. There was a significant decrease in levels of SOD and CAT in plant extract-treated group as compared with the arthritic group as shown in Figure 4.

Table I. Phenolic and flavonoids compound detected in ethanolic extract of *Acacia modesta* in E-AM

Phytochemical	Retention time	Area (m V.s)	Area (%)	Concentration extract (μ g/g)
Quercetin	3.173	40.631	0.9	2.15
Gallic Acid	4.840	463.347	10	16.67
Vanillic acid	13.147	454.290	9.8	28.15
Sinapic acid	26.373	2.702	0.1	0.04
Syringic acid	16.887	125.916	2.7	3.15
Ferulic acid	22.040	73.616	1.6	5.30
Cinnamic acid	25.087	25.421	0.5	0.89
Benzoic acid	14.573	125.418	2.7	13.29
Caffeic acid	12.740	210.006	4.5	9.66

Phytochemical analysis

The qualitative phytochemical analysis of E-AM was carried out by following standard procedures. Saponins,

phenols and flavonoids pool were found in the extract. Results indicated the presence of secondary metabolites in different fractions. The quantitative analysis of the plant extract was carried out by HPLC. It was noted that quercetin, benzoic acid, Gallic acid, vanillic acid, sinapic acid, ferulic acid, syringic acid and cinnamic acid were detected in ethanolic extract of plant as shown in Table I and Figure 5.

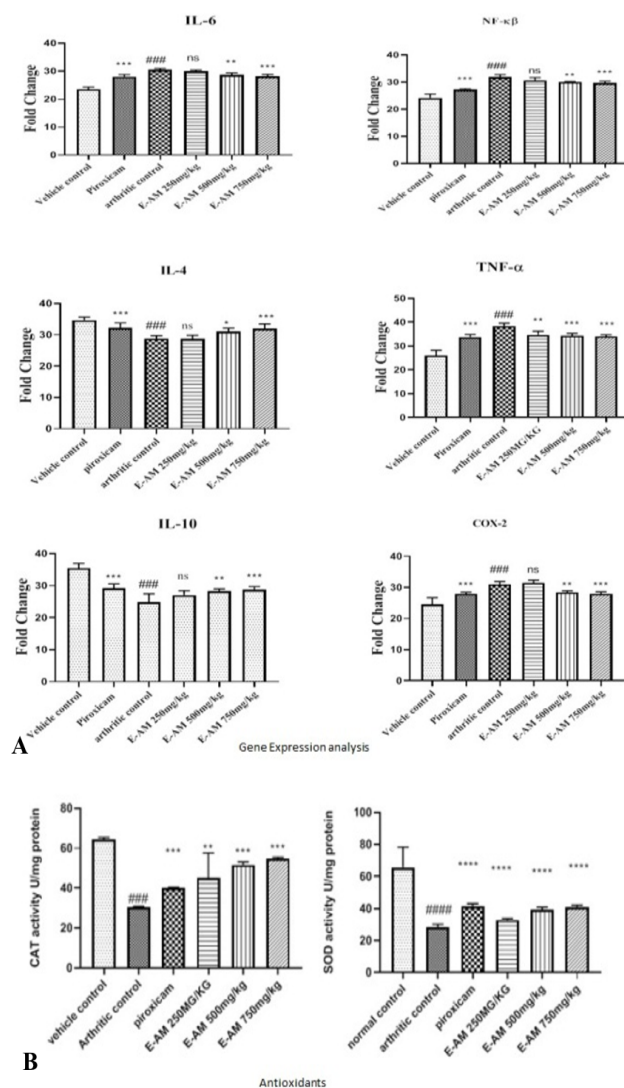


Fig. 4. Effect of different concentrations of ethanolic extract of *Acacia modesta* on gene expression analysis (A), and antioxidative potential superoxide dismutase and catalase (B). The gene expression of several inflammatory biomarkers were evaluated following 28 days of study in wistar rats for mRNA expression of IL4, IL6, IL10, NF- κ B1, TNF- α , COX-2. In case of antioxidants SOD and CAT there was a significant decline of antioxidants compared to arthritic group. ***, **, * showing $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$.

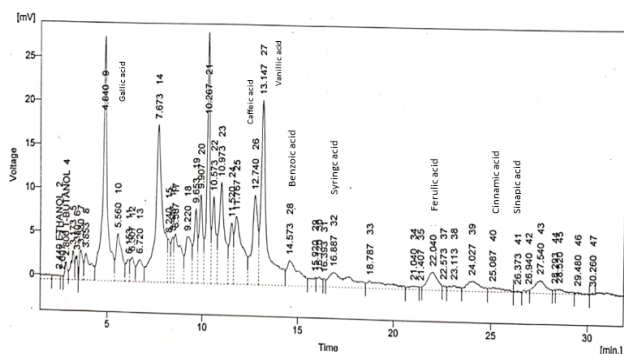


Fig. 5. HPLC peaks of various natural compounds with their retention time found in E-AM extract.

DISCUSSION

Previous research has revealed that therapeutic components/ constituents derived from plant source possess remarkable medicinal potential when extracted out with the aid of organic solvents (Madane *et al.*, 2013). This gives an authentic ground for utilizing different organic solvents for the purpose of extracting therapeutic moieties. Moreover, plants with high content of phytoconstituents are reported to yield a pivotal role in exhibiting their biological effects. Among the key components, flavonoids hold exceptionally great antioxidant potential along with excellent inhibitory effect on prostaglandins synthesizing enzymes which includes protein tyrosine kinase, cyclooxygenases, and phospholipase A2. Phenols and glycosides are believed to have anti-inflammatory activity through the inhibition of reactive oxygen species (ROS) and iNOS pathways. Saponin help in reducing oedema rate by means of depressing vascular infiltration triggered by swelling agents. However, steroids prevent pro-inflammatory cytokines recruitment process like iNOS, COX-2 and TNF- α at wound location thereby favouring anti-oedematous effect (Andreicut *et al.*, 2018). Further findings related to phytochemical qualitative screening exposed that E-AM contained such biologically active fractions which anticipate their role in anti-inflammatory and anti-arthritic activity of both extracts. ROS are held responsible for endothelial dysfunction as well as tissue injury through signalling molecules in inflammatory diseases (Andreicut *et al.*, 2018). Additionally, antioxidants lower down the inflammation process and prevent subsequent cell injury by restraining oxidative stress. Carrageenan oedema is devoid of systemic effects thus glucocorticoids or prostaglandins antagonist serves as a backbone of therapy (Mittal *et al.*, 2014). The current study exhibited positive inhibitory action of ethanolic extract against pro-inflammatory mediators' secretion

which is accountable for its acute anti-inflammatory action. For the estimation of anti-inflammatory effects of test drugs in cases of chronic inflammation in rats, CFA induce oedema is a precise model due to their pathological similarity with human arthritis. In Freund's adjuvant arthritis (CFA) model, oedema reaction appears locally in first ten days followed by subsequent chronic systemic effect which lasts for months (Mittal *et al.*, 2014).

In the present study, animals were given treatments with ethanolic extract and they reduced arthritic index significantly after twenty-eight days of treatment comparative to the arthritic control group which showed decline in the progression of disease. Furthermore, assessment of serum biochemical and haematological parameters in addition with inflammatory biomarkers (CRP, NF- κ B1, interleukins, COX-2 and TNF- α) mainly categorize inflammatory condition of the patient (Patil *et al.*, 2019). Increased levels of alkaline phosphate, ALT, and AST are associated with hypophosphatemia and hepatocellular injury respectively (Olago-Rakuomi, 2017). While in the present work, levels of WBCs, RBCs, platelets, CRP, AST ALP, and ALT expressed no alteration in animals given extract and standard treatments so, it presented the minimum supportive environment for the inflammatory process. However, WBCs and platelets were observed with high levels in the arthritic group which could specify production of immune responses against the pathogens (Foyet *et al.*, 2015). Whereas, IL-4 and IL-10 inhibits Th-1 cell activity by suppressing IFN- γ thus, generate a direct inhibitory effect on macrophages activity in synovium of arthritic patients (Lubberts and van den Berg, 2013). The outcomes of present study revealed expansion in IL-4 and IL-10 levels in both standard and extract-treated groups representing their excellent inhibitory effects against pro-inflammatory cytokines. Moreover, a rapid and severe joint destruction and inflammation, hypercoagulability and hypoalbuminemia were generated when neutrophils excrete protease enzymes and reactive oxygen intermediates. Elevated level of IL-6 was observed in diseases control group during CFA model assessment, while in treated group the levels were not increased and showed a normal range which is accounted for anti-arthritic actions of *ethanolic extracts* and piroxicam. IL-1 β has a key role in cartilage devastation and T-cell activation through the production of reactive oxygen species and proteolytic enzymes while nuclear factor-kappa (I-kB) is responsible for the release of toll like receptor (TLR) pathway by means of inflammatory cytokines (Mateen *et al.*, 2017).

Results of present work displayed reverting in the levels of IL-1 β after standard and extract treatments. This provides the evidence for the inhibitory potential of IL-

1 β towards pro-inflammatory mediator's release. COX-2 stimulates the production of prostaglandins (PGE₂) which in turns activates the release of TNF- α and IL-1 β from chondrocytes thus eventually inducing inflammation and pain in RA. In the present work, real-time PCR expression analysis showed reduction in the levels of COX-2 in ethanolic extract of plant, and standard thereby reduced symptoms of polygenetic arthritis. Various pathological stimuli like oxidative stress, cytokines and growth factors promote NF-kB production and activate the inflammatory environment (Makarov, 2001). A similar mechanism was examined in the synovial lining of CFA-induced arthritic control rats. Our results demonstrated reduction in the levels of NF- κ B1 in groups treated with the ethanolic extract and standard drug. TNF- α is a pro-inflammatory cytokine secreted from monocytes, macrophages, and fibroblasts. It has a dual function either as autocrine or paracrine inducer for other inflammatory mediators (interleukin-1, IL-6, IL-8, GM-CSF) and adhesion molecules (ICAM-1) thus evoking rheumatoid synovitis (Vasanthi *et al.*, 2007). A similar response was seen in arthritic control group however, a notable decline in TNF- α expression was exposed in ethanolic extract and piroxicam treated groups. C-reactive protein is a non-specific and acute inflammatory phase detector with a normal range of 0–8 mg/L whereas; overexpression of CRP (100 mg/L) specifies active rheumatoid arthritis (Finney and Thwaites, 2010). The current study showed normal levels of CRP in treatment groups (E-AM and Piroxicam) which were comparable with anti-inflammatory attributes of plant extract. While, histopathological scoring and microscopic examination of rats paws showed minimum bone erosion and inflammation in ethanolic extract treated groups.

CONCLUSION

The findings from *in vitro* and *in vivo* experimentation revealed that the extract of *A. modesta* possesses beneficial anti-inflammatory and anti-arthritic actions in animal model that may have potential to be an appropriate candidate for the treatment of inflammation. Additionally, *A. modesta* is capable of causing down-regulation in the expression of inflammatory cytokines including, TNF- α , IL-6, COX-2, IL-1B, and NF- κ B1 along with up-regulation of IL-4 and IL-10 anti-inflammatory biomarkers. Further investigations regarding the potential application of E-AM extracts in arthritic patients and safety evaluations, are yet to be studied; these will be needed to assess the feasibility and safety of any E-AM extracts based clinical interventions.

Conflict of interest statement

The authors have no relevant financial or non-financial interests to disclose.

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